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Original Contribution

3,3′-Diindolylmethane decreases VCAM-1 expression and alleviates experimental colitis via a BRCA1-dependent antioxidant pathway

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ABSTRACT

Reactive oxygen species (ROS) exhibit a key role in the pathogenesis of inflammatory bowel disease (IBD). 3,3′-Diindolylmethane (DIM) can protect against oxidative stress in a breast cancer susceptibility gene 1 (BRCA1)-dependent manner. The aim of this study was to examine the therapeutic effects of DIM in experimental colitis and investigate the possible mechanisms underlying its effects on intestinal inflammation. The therapeutic effects of DIM were studied in 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis. Pathological markers of colitis severity, antioxidant activity, and ROS generation in colonic tissue were measured. The impact of DIM on ROS-induced endothelial vascular cell adhesion molecule 1 (VCAM-1) expression and leukocyte–endothelial cell interaction was further investigated in cultures of endothelial cells and in the TNBS-induced colitis model. Administration of DIM was demonstrated to attenuate experimental colitis, as judged by pathological indices. DIM could effectively stimulate the expression of BRCA1 in vitro and in vivo and reduce ROS generation, leading to the inhibition of VCAM-1 expression and leukocyte–endothelial cell adhesion, and finally resulted in an alleviation of experimental colitis. DIM has shown anti-IBD activity in animal models by inhibiting ROS-induced VCAM-1 expression and leukocyte recruitment via a BRCA1-dependent antioxidant pathway and thus may offer potential treatments for IBD patients.

Introduction

3,3′-Diindolylmethane (DIM) is a natural product formed during the autolytic breakdown of glucobrassicin present in food plants of the *Brassica* genus. DIM is a promising anti-tumor agent that has been widely studied in laboratory and clinical trials [1–3]. Moreover, recent studies demonstrated that DIM could protect against reactive oxygen species (ROS) in a breast cancer susceptibility gene 1 (BRCA1)-dependent manner [4].

The inflammatory bowel diseases (IBD), including Crohn disease and ulcerative colitis, are chronic inflammatory disorders of the gastrointestinal tract [5]. There is substantial evidence that ROS exhibit a key role in the pathogenesis of IBD [6–8]. ROS can induce a quantitative up-regulation of endothelial vascular cell adhesion molecule 1 (VCAM-1), because several oxidant-sensitive transcription factors are associated with activation of VCAM-1 gene expression [9,10]. In IBD, increased expression of VCAM-1 on intestinal vascular endothelium modulates leukocyte firm adhesion to endothelial cells and migration to sites of inflammation [11,12]. The dense intestinal infiltrate of inflammatory cells exhibits a distinct distribution for Crohn disease as well as ulcerative colitis [13,14].

Based on these observations, this study was designed to evaluate whether treatment with DIM exerts protection on established experimental colitis induced by trinitrobenzene sulfonic acid (TNBS). Furthermore, we investigated the possible mechanism involved in DIM's anti-IBD activity: DIM may confer protection by its effects on VCAM-1 expression and leukocyte recruitment via enhanced expression of BRCA1.

Methods

Reagents

DIM was purchased from Ruima Chemical Co. (Nanjing, China; purity >99%). DIM is not a water-soluble compound. An oil-dissolved

Abbreviations: Ahr, aryl acetylcholine receptor; ARE, antioxidant response element; BRCA1, breast cancer susceptibility gene 1; DAI, disease activity index; DIM, 3,3′-diindolylmethane; GST, glutathione S-transferase agents; HUVEC, human umbilical vein endothelial cells; IBD, inflammatory bowel diseases; IL, interleukin; MPO, myeloperoxidase; NRF2, nuclear factor erythroid-derived 2-like 2; ROS, reactive oxygen species; SOD, superoxide dismutases; TNBS, 2,4,6-trinitrobenzene sulfonic acid; T-AOC, total antioxidative capacity; VCAM-1, vascular cell adhesion molecule 1.

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DIM formulation was used in previous experiments in cells and was orally given in animal experiments [15,16]. When this kind of formulation is injected in vivo, the DIM-containing oil drops is captured mainly by cells of the reticuloendothelial system and hardly enters other tissues and cells. To solve this problem, DIM was formulated in 2-hydroxypropyl-β-cyclodextrin to enhance its water solubility according to our previous studies [17]. Briefly, 20 mg DIM was dissolved in 1 ml 2-hydroxypropyl-β-cyclodextrin (Sigma–Aldrich, St. Louis, MO, USA) solution (molar ratio 1:10; prepared at 40 °C for 20 min). These stock solutions (20 mg/ml) were serially diluted in sterile water to give final concentrations (2 or 10 μmol/L for in vitro experiments and 5 mg/ml for in vivo experiments) on the day of use. This formulation of DIM can be injected into animals rather than being orally given, which can help to obtain more stable and credible experimental results when investigating its pharmacological mechanisms. RPMI 1640, M199, penicillin/streptomycin antibiotic mixture, and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA, USA). Lipopolysaccharide was purchased from Sigma–Aldrich. Other chemical reagents were purchased from Sangon Biotech (Shanghai, China).

Cell culture

Human umbilical vein endothelial cells (HUVEC) were harvested from umbilical cords by collagenase treatment (Sangon Biotech, Shanghai, China) as previously described [18]. HUVEC were grown in M199 supplemented with 10% FBS. Cells were used after the second or third passage for all experiments. RAW 264.7 cells (ATCC, Manassas, VA, USA) were cultured in RPMI 1640 containing 10% FBS. The cell cultures were incubated in room air with 5% CO_2 at 37 °C and 95% humidity.

Cell treatment

In the experiments to evaluate the antioxidant effect of DIM, lipopolysaccharide (LPS; 1 μg/ml) was added to the cell plates for 24 h in the presence of DIM (10 μmol/L). After LPS treatments, cells were collected and homogenized. BRCA1 expression level in HUVEC was determined by real-time PCR. To test the role of BRCA1 in DIM-mediated antioxidant activity in cells, HUVEC were pretreated with BRCA1 small interfering RNA (siRNA) or control siRNA (50 nmol/L) for 24 h before incubation with DIM/LPS. BRCA1 siRNA and control siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Glutathione *S*-transferase agent (GST) activity and total antioxidative capacity (T-AOC) of cell samples were analyzed using commercial kits (Jiancheng Biotech, Nanjing, China). As previously described [19], the ferric reducing/antioxidant power assay was used to determined T-AOC. The principle of this method is based on the reduction of a ferric–tripyridyl triazine complex to its ferrous colored form in the presence of antioxidants.

Expression of VCAM-1 and leukocyte adhesion assay

To investigate the effect of DIM on ROS-treated cells, HUVEC were exposed to H_2O_2 (100 nmol/L) in the presence of various concentrations of DIM (2 or 10 μmol/L) for 24 h. For the adhesion assay, RAW 264.7 cells used to imitate leukocytes were suspended in phosphate-buffered saline (PBS) buffer and fluorescence labeled with carboxyfluorescein succinimidyl ester (Invitrogen). RAW 264.7 cells (10^6/well) were added to the pretreated HUVEC and 15 min later the unbound cells were removed by four washes with PBS. Cell plates were examined by fluorescence microscopy (TE2000-U; Nikon, Tokyo, Japan). Adherent RAW 264.7 cells were lysed by 1% Triton X-l00 (Sangon Biotech) and quantified by microplate reader (Tecan Group Ltd, Männedorf, Switzerland). The VCAM-1 expression level in HUVEC was determined by real-time PCR and immunofluorescence staining.

Establishment of experimental colitis and treatment

Female BALB/c mice of the same background were obtained from the Laboratory Animal Center of Nanjing University (Nanjing, China). All animals received humane care according to Chinese legal requirements.

TNBS-induced colitis was conducted in 6- to 8-week-old female BALB/c mice as reported previously [20,21]. Briefly, 2.5 mg of TNBS (Sigma) in 100 μl 50% ethanol was slowly administered into the lumen of the colon via a catheter inserted 4 cm into the colon through the anus. Mice were then kept in a vertical position for 30 s. Control mice received 50% ethanol alone.

DIM was given daily after the induction of colitis in the TNBS-induced experimental model at different doses via intraperitoneal injection with 2-hydroxypropyl-β-cyclodextrin at the same concentration as the control. The mice were checked daily for behavior and body weight.

Assessment of colonic inflammatory changes

To investigate the therapeutic effect of DIM, mice were sacrificed at day 3 after colitis induction in the experimental model. The colons

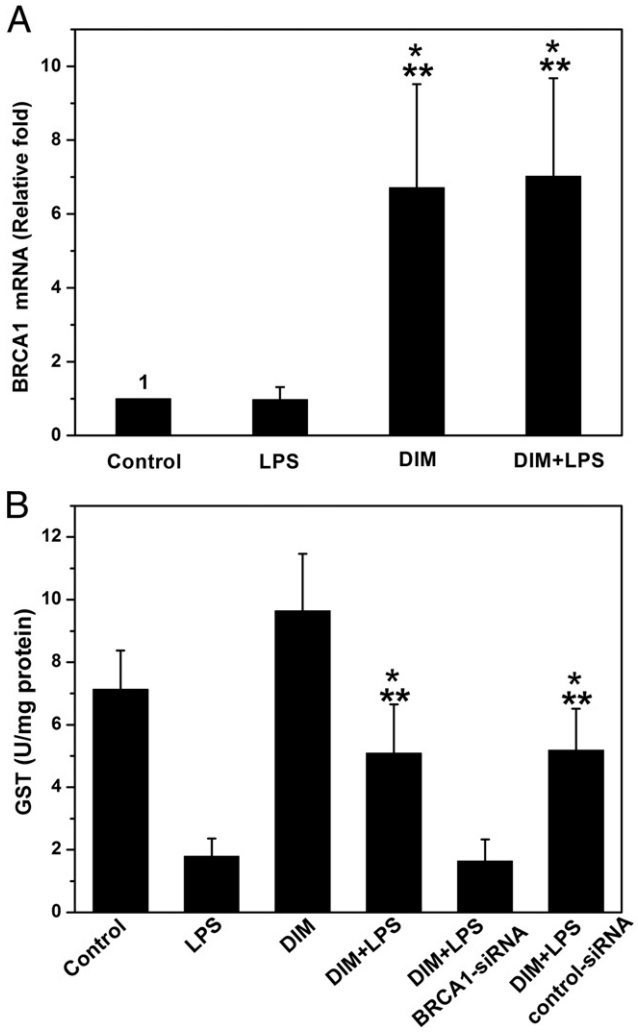

Fig. 1. Antioxidant activity of DIM in endothelial cells. (A) After treatment with 10 μmol/L DIM and 1 μg/ml LPS for 24 h, the BRCA1 expression level in HUVEC was examined. (B) HUVEC were pretreated with BRCA1 siRNA, control siRNA (50 nmol/L × 24 h), or no siRNA; incubated with DIM (10 μmol/L) or vehicle plus LPS for 24 h; and harvested for GST assays. Values are expressed as means ± SEM of four or five samples per condition. $^{*}p \le 0.05$ compared with control HUVEC, $^{**}p \le 0.05$ compared with LPS-treated HUVEC (A). $^{*}p \le 0.05$ compared with LPS-treated HUVEC, $^{**}p \le 0.05$ compared with DIM and BRCA1 siRNA-treated HUVEC (B).